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From Cell Biology to Tissue Engineering

Different effects of olive leaf extract on antioxidant enzyme activities in midbrain and dopaminergic neurons of Substantia Nigra in young and old rats

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Summary. Objectives: Study of the effects of olive leaf extract on antioxidant enzyme activities in midbrain and dopaminergic neurons of Substantia Nigra in young and old rats. Methods: Male wistar rats age 4 and 18 months were randomized into control and experimental groups. A single daily dose of 50 mg/kg of olive leaf extract was administered orally by gavage to each rat for 6 months. The control group received only distilled water. All rats were sacrificed 2 hours after the last gavage and their midbrains were separated for Malondialdehyde (MDA) and antioxidant enzyme activitiy analysis. TUNEL assay and immunohistochemical (IHC) staining were used for evaluation of the number of neurons in the Substantia Nigra. Results: The level of Catalase, Glutathione Peroxidase and Superoxide Dismutase enzyme activity were significantly increased in experimental young and old groups compared to their control groups. However the level of Superoxide Dismutase enzyme activity was significantly increased in experimental old group when compared to control group ($P<0.01$), the level of Superoxide Dismutase enzyme activity was not significantly changed in young groups. MDA level was decreased significantly in experimental young and old rats compared to their control groups. Histological analysis demonstrated that the number of neurons in Substantia Nigra of experimental old group was more than the control group ($P<0.01$). The number of apoptotic cells was significantly decreased in experimental old group compared to the corresponding control group ($P<0.05$). In IHC and TUNEL assay, no change was observed in the number of neurons between experimental and control young groups. Conclusion: Long term treatment with olive leaf extract increases antioxidant enzyme activity and protects the neurons in Substantia Nigra against oxidative stress.

Key words: Olive leaf extract, Substantia Nigra, Midbrain

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Introduction

Oxidative stress is a major factor for lipid peroxidation in the brain. Excessive production of Reactive Oxygen Species (ROS) and decline of antioxidant enzyme activity result in oxidative stress. Elevated oxidative stress interferes with the function of neurons (Floyd and Carney, 1992; Dringen, 2000). Recently, attention has been drawn toward dietary strategies that can protect brain tissue against oxidative stress damage. Polyphenol compounds possess antioxidant and anti-inflammatory properties (Youdim et al., 2002). The neuroprotective effect of olive phenols upon oral administration in mice has been shown and olive leaf extract is rich in phenols including Oleuropein and Hydroxy Tyrosol (HT) (Visioli et al., 1999). Its medicinal use in ancient Egypt was reported (Durlu-Ozkaya and Ozkaya, 2011). Oleuropein is the main glycoside present in olive leaf and Hydroxy tyrozol the

derivative of oleuropein is effective in prevention of complications associated with oxidative stress (Visioli et al., 2002). Recent studies have also indicated that HT exerts its neuroprotection effect after long term oral administration of olive leaf extract in mice (Gonzalez-Correa et al., 2008). Therefore, it is believed that HT reduces the neuronal damage induced by oxidative stress (Schaffer et al., 2007). In the present study, we investigated the neuroprotective effect of olive leaf extract on midbrain by measuring lipid peroxidation level, antioxidant enzyme activity and also by histological examination of Substantia Nigra (SN) tissues in young and old rats.

Materials and methods

Olive leaf extract was provided from Razi herbal medicine research center (Lorestan, Iran). Male Wistar rats age 4 months (220-240 gr) and 18 months (450-550 gr) were housed in a temperature controlled room at 23°C and 12 hours light and dark cycle. The animals were fed with pellets during the experiment and randomized into four groups, each containing 10 rats. The four groups were as follows; 1- Control young rats 2- Experimental young rats 3- Control old rats 4- Experimental old rats. All animal work was approved by the ethical guidelines for the care of laboratory animals of the research center of Iran University of Medical Sciences (Tehran, Iran).

The experimental groups received a daily single dose of 50 mg/kg of olive leaf extract by oral gavage for 6 months. The control groups received only distilled water. Two hours after the last oral gavage half of the rats from each group were anesthetized and decapitated, their brains were removed and immersed in cold PBS (0.1 M, pH: 7.4) Then their midbrains were separated and homogenized in ice cold 10nmol/l Tris –HCl. The homogenate was centrifuged at 12000 x g at 4°C for 20 minutes. The supernatant was collected for antioxidant enzyme activity assay. Lipid peroxidation level was measured by Thiobarbituric acid (TBA) using the method of Satoh (Satoh, 1978). This method was used to measure the color development that results from the reaction of TBA with Malondialdehyde. Superoxide Dismutase (SOD) activity was measured by the method of Misra and Fridovich (Misra and Fridovich, 1972). Catalase (CAT) activity was measured based on the ability of the enzyme to break down the hydrogen peroxide (H_2O_2). This was performed according to modified version of Aebi method (Aebi, 1984). Glutathione Peroxidase (GPx) activity was determined using a kit from Randox (UK) following the manufacturer's instruction. The other half of the rats were anesthetized and perfused transcardially with PBS (pH: 7.4) followed by fixation in 4% paraformaldehyde. Their brains were removed and further fixed in the same fixative overnight. After incubation, the midbrains were separated and dehydrated in graded concentration of alcohol, cleared in xylene, infiltrated with paraffin and finally embedded in paraffin. The 5 μm coronal sections were serially collected from bregma-4.52 mm to -6.04 mm of midbrains (Paxinos and Watson, 2006) with a 30 μm interval between each consecutive section. Half of the sections were stained for TUNEL assay using a detection kit from Roche, according to the manufacturer's instructions (Roche, Germany). Briefly, the sections were rehydrated, incubated in 3% H_2O_2 for 10 minutes and then incubated in proteinase –K^2 (20 μg/ml in 10 mM Tris/HCL, pH: 7.6) for 30 minutes. After incubation, TUNEL reaction mixture was added to the sections and incubated for 1 hour. Each 5 μm section was incubated further with antibody conjugated horseradish peroxidase (Roch, Germany) for 30 minutes and developed with 0.05% 3,3-Diaminobanzidine (DAB) for 1-2 min and counterstained with Hematoxylin. The cells with brown stained nuclei were counted at a magnification of × 400. For positive control the 5 μm sections were incubated in DNAase (3000 U/ml in 50 mM Tris-HCl, pH: 7.5, 1 mg/ml BSA) for 10 min to induce DNA strand break prior to labeling procedure. For negative control the sections were incubated with labeling buffer only.

For immunohistochemistry staining (IHC), the sections were dehydrated in graded concentration of alcohol, immersed in 10% H_2O_2/methanol for 10 minutes and washed in 0.1 Tris wash buffer (TBS). For retrieving the antigens, the sections were kept in citrate buffer and boiled for 11 minutes. After cooling, the sections were washed in Tris buffer and incubated in Bovine Serum Albumine (BSA) for 10 minutes. After antigen retrieval, the sections were incubated in the primary antibody (mouse monoclonal antibody to tyrosine hydroxylase (1:80, Abcam, UK) for 1 hour at room temperature. The sections were washed in Tris buffer (pH: 7.4) and incubated in HRP conjugated secondary antibody (1:100, Abcam, UK) for 1 hour. They were incubated with DAB for 10 minutes, washed in Tris buffer (pH: 7.4) and counterstained with hematoxyline, the sections were washed under tap water, dehydrated in graded concentration of alcohol, cleared in Xylol and covered with a cover slip. The cells were counted in five coronal sections of substantia nigra from each animal in five separate microscopic fields randomly with ×400 magnification using a microscope (Olympus AX70), Olympus DP11microscope digital camera and OLYSIA autobioreport software (Olympus optical Co.Ltd.Japan). A grid was superimposed on the photographs and the cells with obvious nucleus were counted, then a cross was placed on the counted cells to prevent recounting (Mehraein et al., 2011). Statistical analyses were performed using SPSS by t-test and ANOVA. The data are expressed as mean ± standard deviation (SD).

Results

Fig. 1A shows SOD activity in all groups. SOD activity was increased in experimental old group

compared to corresponding control group (P<0.01). An increase of SOD activity was also observed in experimental young group compared to their control, which statistically was not significant. Administration of olive leaf extract caused a significant increase in CAT activity in midbrain tissues of experimental old rats when compared to their control group (P<0.01), as shown, the level of CAT enzyme activity in experimental old group reached the same level of CAT enzyme activity in control young rats (P<0.05) (Fig. 1B). Also, a significant increase of CAT enzyme activity was observed in experimental young group compared to control young group (P<0.05) (Fig. 1B). A marked increase of GPX enzyme activity was observed in experimental old group when compared to their corresponding control group (P<0.01) as well as in experimental and control young groups (P<0.05) (Fig. 1C). The level of MDA in midbrain tissues of control old rats was significantly higher than the other groups (P<0.05), while the level of MDA in experimental old rats was significantly decreased compared to their control group (P<0.01). There was also a significant reduction of the level of MDA in experimental young group in comparison to control young group (P<0.05) (Fig. 1D). Analysis of TUNEL assay revealed a small number of TUNEL positive neurons in SN of experimental young group compared to control young group but the difference was not significant (Fig. 2C,D).

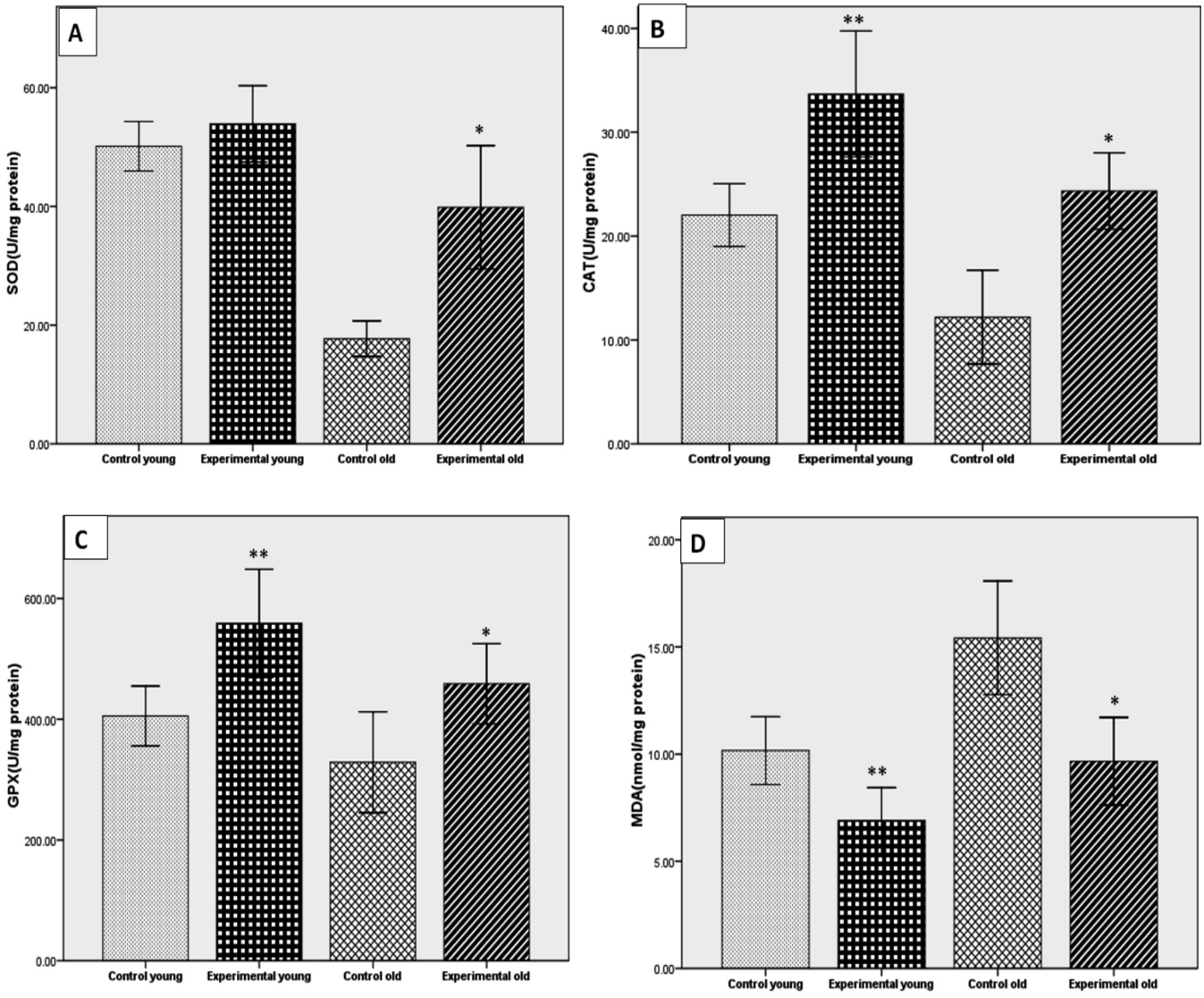
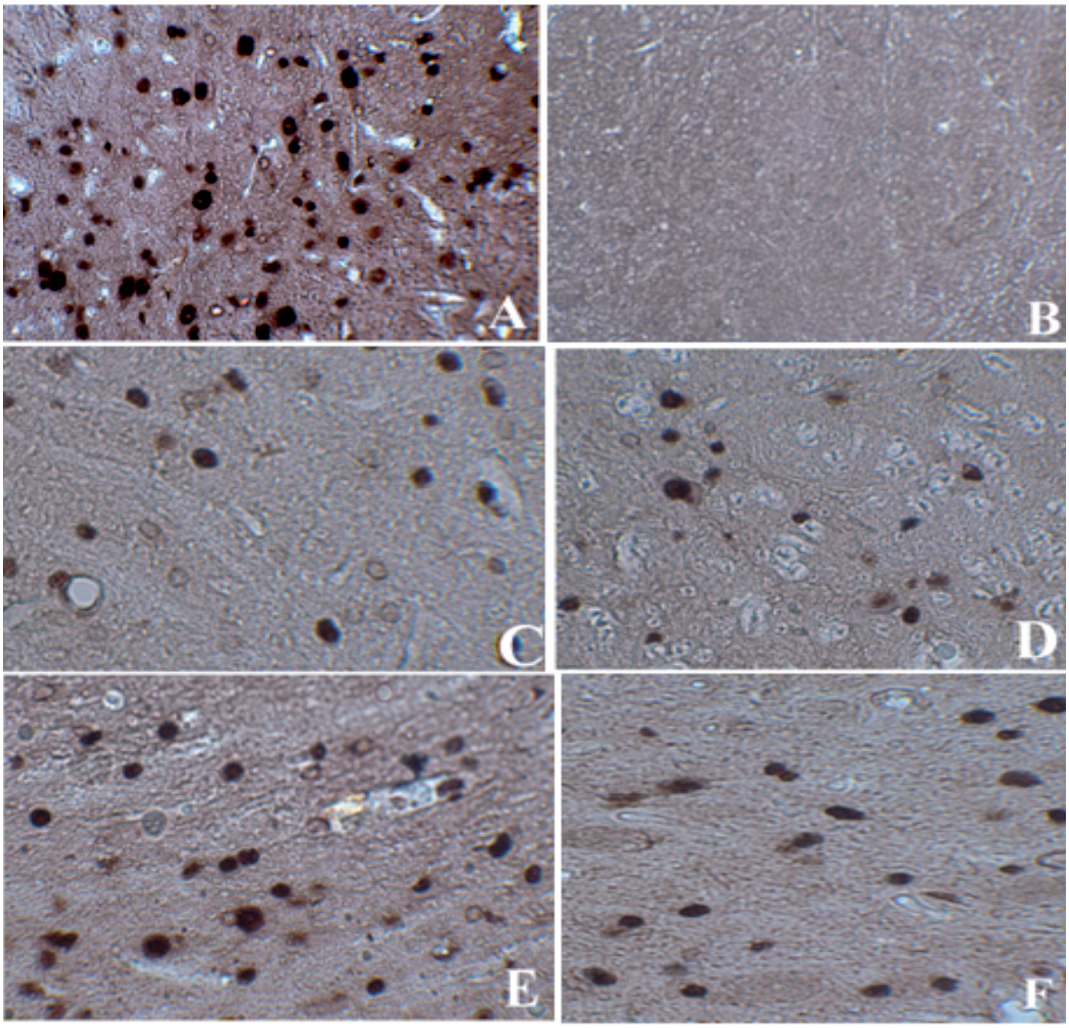


Fig. 1. Antioxidant enzyme activities in control and experimental young and old groups. **A.** Superoxide dismutase (SOD). **B.** Catalase (CAT). **C.** Glutathione peroxidase GPx. **D.** The levels of malondialdehyde (MDA). Values are expressed as mean ± SD. *P<0.01 vs. Control old, **P<0.05 vs. control young.



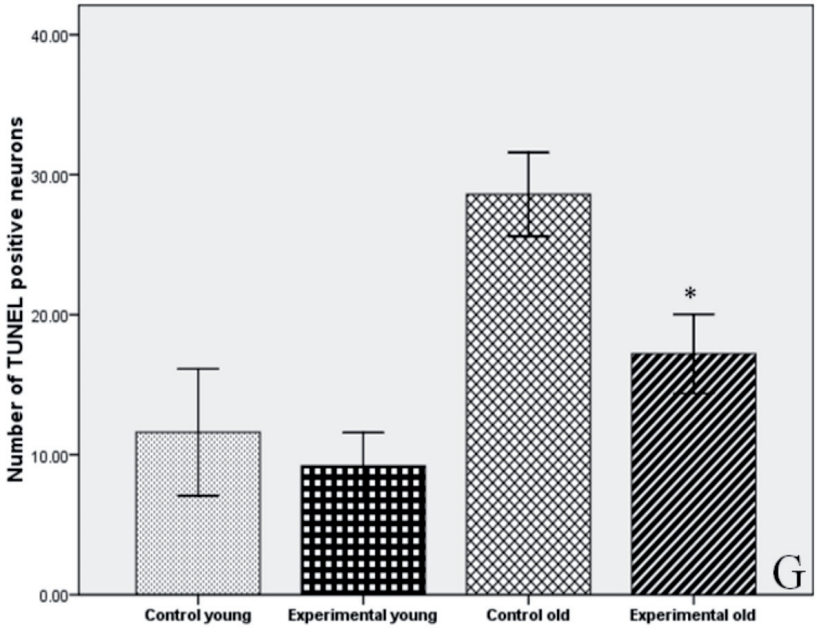


Fig. 2. Photomicrographs of Tunel staining of SN in the control and experimental young and old groups. **A.** Positive control staining. **B.** Negative control staining. **C.** Control young group. **D.** Experimental young group. **E.** Control old group. **F.** Experimental old group. **G.** The histogram shows the differences in the number of apoptotic neurons between control and experimental old groups which was significant (*P<0.05). x 400

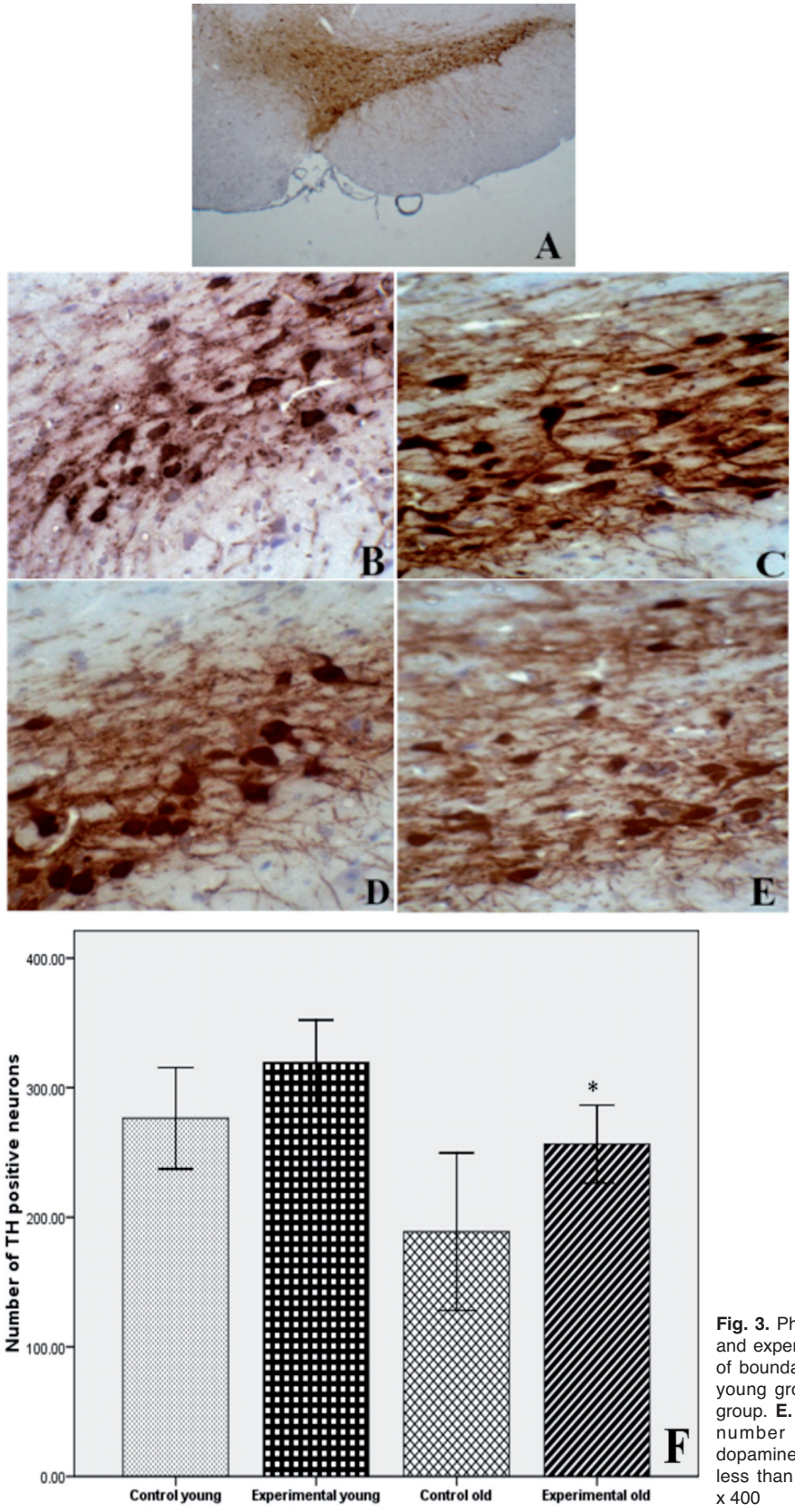


Fig. 3. Photomicrographs of IHC staining of SN in the control and experimental young and old groups. **A.** Photomicrograph of boundaries of the midbrain in coronal section. **B.** Control young group. **C.** Experimental young group. **D.** Control old group. **E.** Experimental old group. **F.** Columns represent the number of dopaminergic neurons. The number of dopaminergic neurons in control old group was significantly less than experimental old group (*P<0.01). A, x 100; B-E, x 400

TUNEL staining also showed that the number of apoptotic neurons in experimental old group was less than the number in corresponding control group ($P<0.05$) (Fig. 2E,F.G). IHC staining of the SN tissues revealed that the differences of the number of TH^+ neurons between experimental and control young groups were not significant (Fig. 3B,C), while experimental old group had significantly more neurons compared to their control group ($P<0.01$) (Fig. 3D,E,F).

Discussion

ROS is produced during normal metabolism which may lead to oxidative damage to lipids and proteins. The excess of ROS that is the result of biochemical changes in neurons may lead to neuronal dysfunction and death of the neuron (Poon et al., 2004). Brain tissue is rich in polyunsatured lipids and has high iron content and its function is dependent on aerobic metabolism, therefore it is vulnerable to oxidative damage (Beckman and Ames, 1998). Malondialdehyde (MDA) level is a marker of lipid peroxidation and oxidative stress (Kasapoglu and Ozben, 2001). Lipid peroxidation caused by ROS is involved in several brain disorders. In this study, a comparative analysis of MDA in control young and control old rats showed a higher level of MDA in control old rats, indicating that the level of MDA increases by age, as has been reported by others (Kasapoglu and Ozben, 2001). In human, the mean MDA values in 50-59 age groups are significantly higher than 30-39 age groups (Kasapoglu and Ozben, 2001). The data from the same study also showed that the level of oxidative stress by aging may be related to a decrease in antioxidant enzyme activity. Protective antioxidant enzymes reduce oxidative stress by degrading the ROS. SOD converts superoxide radicals into hydrogen peroxide and oxygen. CAT and GPX convert H_2O_2 to water and oxygen (Sies, 1993). In the present study, we showed that the level of SOD and CAT enzyme activity in control old rats was less than control young group, which is in agreement with Head and Kasapoglu studies (Kasapoglu and Ozben, 2001; Head, 2009). Our findings also indicated that GPX enzyme activity was not different between young and old control groups, which is consistent with Holmes et al (Holmes, 1999; Erden-Inal et al., 2002). In contrast Sandhu reported the decrease of GPX enzyme activity in old subjects compared to young group (Sandhu and Kaur, 2002). There are reports that show the neuroprotective properties of olive phenolic compounds on reduction of lipid peroxidation in neurons (De La Cruz et al., 2000). It is believed that a diet rich in antioxidant may reduce oxidative damage to neurons during aging (Elmadfa and Meyer, 2008). In this study, we observed a significant decrease in MDA level in the experimental young and old rats. Our results also showed that CAT and GPX enzyme level were significantly increased in the experimental young and old groups compared to their control groups. SOD enzyme activity was increased significantly in experimental old group when compared to corresponding control group; however the differences in young groups did not reach a statistical significance. SOD enzyme activity causes morphological and biochemical changes (Pejic et al., 1999). Studies showed that olive leaf extract scavenges free radicals by inducing antioxidant enzyme activity (Visioli et al., 2002). Olive phenolic compound primarily may increase the activity of antioxidant enzymes in the brain to prevent free radical oxidative damage (Servili et al., 2014). We observed that apoptotic cell numbers in control old rats are significantly more than control young group. The results from IHC staining also showed that control old rats had less dopaminergic neurons than control young rats in SN of their midbrain as reported before by Gao (Gao et al., 2011). Analysis of TUNEL assay revealed that the number of apoptotic neurons was reduced in experimental old group compared to their control. These data was supported by IHC staining which showed that the number of TH positive neurons in experimental old group was significantly more than control old group while the differences of the number of TH positive neurons in experimental and control young groups were not significant.

Conclusion

Dietary supplementation of olive leaf extract plays an important role in prevention of neuron loss in SN and increases the antioxidant enzyme activity against oxidative damage.

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